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Award Number: W81XWH-09-1-0211

TITLE: Identification of Genes Required for the Survival of Prostate Cancer Cells

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REPORT DATE: June 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-06-2011	Annual	15 MAY 2010 - 14 MAY 2011

4. TITLE AND SUBTITLE

Identification of Genes Required for the Survival of Prostate Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-09-1-0211

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Brigham and Women's Hospital, Inc.
Boston, MA 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Prostate cancer, specifically castration-resistant prostate cancer (CRPC), is the second leading cause of cancer-related deaths among men in the United States. The manifestation of prostate tumors capable of evading androgen-dependency results in highly aggressive metastatic prostate cancers that become effectively incurable due to the absence of treatments targeting alternative pathways. To identify novel therapeutic targets distinct from those essential for androgen-dependent prostate tumor survival and proliferation, we have employed a somatic cell genetics approach in the form of a comparative loss-of -function genome-wide shRNA screen. To date, we have identified a family of nuclear hormone receptors as being critically required for prostate cancer growth in culture. We expect our research study, once fully completed, to facilitate the future identification and development of small molecule inhibitors to these potential prostate cancer drug targets.

15. SUBJECT TERMS

None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	21	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Prostate cancer, specifically castration-resistant prostate cancer (CRPC), is the second leading cause of cancer-related deaths among men in the United States. The manifestation of prostate tumors capable of evading androgen-dependency results in highly aggressive metastatic prostate cancers that become effectively incurable due to the absence of treatments targeting alternative pathways. To identify novel therapeutic targets distinct from those essential for androgen-dependent prostate tumor survival and proliferation, we have employed a somatic cell genetics approach in the form of a comparative loss-of -function genome-wide shRNA screen. To date, we have identified a family of nuclear hormone receptors as being critically required for prostate cancer growth in culture. We expect our research study, once fully completed, to facilitate the future identification and development of small molecule inhibitors to these potential prostate cancer drug targets.

Body

Background and Significance

Prostate adenocarcinoma is the most frequently diagnosed cancer and second leading cause of cancer-related deaths among men in the United States, accounting for an estimated 25% of all new cases and 10% of all cancer-related fatalities in 2008 (*1*). The risk of prostate cancer increases significantly with age, as is the case with most cancers, and the rate of incidence is expected to increase as a result of an aging baby boom generation (*2*).

Although the 5-year survival rate of diagnosed prostate cancers is over 90% (*1*), long-term survival is bleak. This is due to the fact that unless the disease has been completely surgically removed, most tumors return as aggressive, androgen-independent (AI), metastatic cancers (*3*). In contrast to localized prostate tumors, metastatic prostate cancer has only a 32% 5-year survival rate (*4*). For sustained growth and proliferation both normal and transformed prostate cells require androgens that activate androgen receptor (AR), a transcription factor that acts as a master regulator of G1-S phase progression (*5*). Current androgen ablation therapies either severely reduce testosterone production by removing the testes, or inhibit its release by administering agonists or antagonists of factors that lie directly upstream of testosterone secretion, such as luteinizing hormone-releasing hormone (LHRH) (also known as gonadotropin-releasing hormone – GnRH). This is often done in combination with inhibitors of adrenal steroidogenesis (*2*). Alternative therapies, such as estrogen administration, pure steroidal and non-steroidal direct competitive inhibitors of dihydrotestosterone (DHT), and 5α-reductase inhibitors have proved less successful (*2*).

Unfortunately, androgen ablation has been shown in several studies to be overcome by a positive selection for AI cells (*6-8*), which arise through a variety of mechanisms, including mutations that change AR specificity, sensitivity, and/or expression at a frequency of up to 50% in metastatic prostate tumors (*3*). Moreover, overexpression of insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), human epidermal growth factor-2 (HER-2/neu), and c-myc (*9-12*) can inappropriately activate AR, while tumor suppressors such as PTEN, pRb, p53 are frequently inactivated (*13*). Bcl-2, Bcl-xL, and clusterin pro-survival genes have also been implicated in the development of AI tumors (*14-16*). Additionally, because prostate cancers are heterogeneous, androgen-independent cells already present in the primary tumor may be selected for over the course of androgen ablation treatment (*3*).

The complexity of AI tumors and the universal degree to which they arise as a result of androgen ablation treatments make it critical to identify alternative targets for treatment. Although radiation and chemotherapeutic treatments have shown promise in increasing survival and time of relapse (*1*), the only drug targets exploited to date involve pathways that control androgen production and activity. Clearly, the rate-limiting process for

development of new prostate cancer therapies is target discovery. Exploiting the genetic and epigenetic differences between cancer and normal cells is a universal approach to identify new targets for cancer therapeutics, though this fundamental method has proven challenging. Sequencing efforts have revealed vast numbers of alterations in tumors, but at this point it is difficult to determine which perturbations are causative or simply benign passengers. Gain-of-function mutations in oncogenes represent the most promising targets for future drug development because of the "oncogene addiction" phenomenon, where cancer cells become abnormally dependent on the function of the oncogenes that drive tumorigenesis (*17*). In addition, we have proposed a dependency termed non-oncogene addiction in which tumors are dependent upon non-oncogene targets (*18*).

For example, there is evidence of significant AR crosstalk with growth factor receptor, MAPK, cytokine, and other signaling pathways that are implicated in prostate cancer progression, and mediators of these pathways represent non-oncogene targets for prostate cancer treatment (*19*). In addition there are likely to be other targets of which we are completely unaware that could cause systems failure in the presence of a prostate cancer network of mutations. Signaling mediators that support either of these types of oncogenic and non-oncogenic pathways are ideal targets for cancer therapeutics. However, in the case of non-oncogene addiction these genes will not be mutant in tumors and therefore will be missed by sequencing approaches alone.

A major focus of my lab has been the development and application of technologies for functional genomics in mammalian cells, with a particular focus in the area of cancer biology. With our collaborators in Greg Hannon's laboratory at Cold Spring Harbor Laboratories, we have developed bar-coded shRNA libraries in retroviral vectors and Orfeome expression libraries that allow us to perform comprehensive screens for cancer relevant genes. The shRNAs become processed through the endogenous miRNA pathway and are robustly driven by a strong Pol-II promoter (*20,21*). Both enrichment and dropout screens require methods for "deconvoluting" the screen or pinpointing the bioactive clones. Using the shRNA sequence itself as a barcode, (the half-hairpin barcode) in addition to a unique 60-mer sequence in each shRNA clone, we can achieve multiple read-outs for each single vector on the microarray (**Figure 1**) (*22*). To date, we have performed the desired drop-out shRNA screens to look for genes required for cancer cell proliferation and survival (*22*) in addition to positive enrichment RNAi screens for checkpoint defects (*23*), cellular transformation (*24*), and ubiquitin ligases for tumor suppressors (*25*). Thus, our screening technology is a powerful and complementary approach to large sequencing efforts and is expected to provide many potential cancer drug targets (*22,26*) in an unbiased fashion, essentially asking the cancer cell to direct us to appropriate targets.

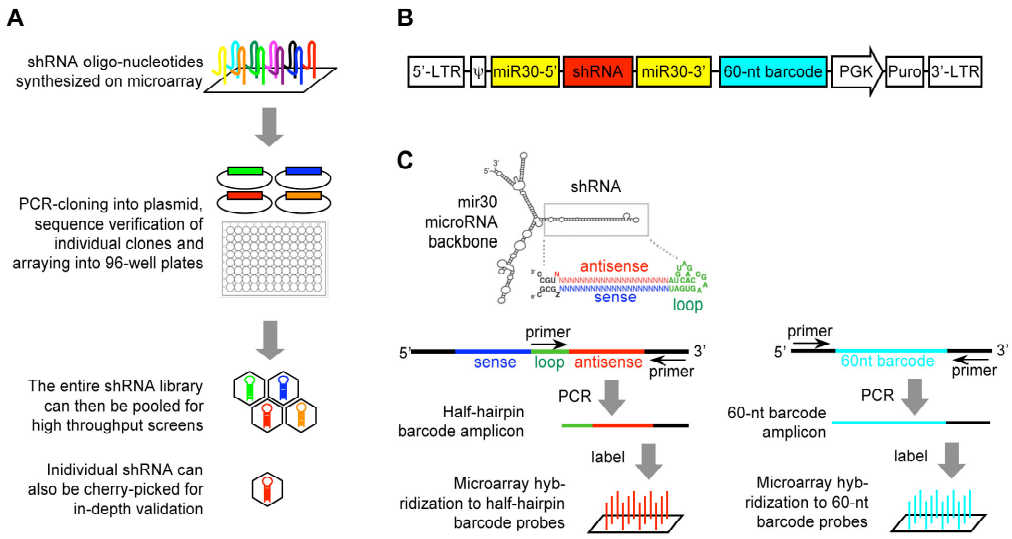


Figure 1. Overview of the Elledge-Hannon barcoded shRNA library.
A. Schematic of library construction. **B.** Schematic of the retroviral vector used to potently express the shRNA library. The shRNA is embedded in the backbone of a naturally occurring microRNA (mir30) to facilitate shRNA expression and maturation. The vector carries a puromycin-resistance marker for selection. **C.** Schematic of half-hairpin (HH) barcode and 60-nt barcode deconvolution. Because the HH and 60-nt barcodes are unique for each shRNA, the abundance of individual shRNAs in a complex pool can be tracked by hybridizing their barcode to a microarray containing the appropriate probes.

Comparative genome-wide shRNA screening for PCL genes

We have hypothesized that genetic loss-of function screens, in combination with bioinformatic and genomic analyses, can identify new targets for prostate cancer therapeutics. Using the strategies that we outlined in our original proposal, we have identified a number of potential genes that are selectively required for proliferation and/or survival of prostate cancer cells but not normal prostate epithelial cells, which we have termed Prostate Cancer Lethal (PCL) genes.

Comparative genome-wide shRNA screen identifies a subfamily of nuclear hormone receptors as potential PCL genes

To identify PCL genes, shRNAs can be screened either in a pool or in an individual, well-by-well format. Although pooled screening is advantageous over the latter for its highly-parallel nature and reduced cost and effort, the relative abundance of thousands of shRNAs in a pool must be measured simultaneously. Utilizing each shRNA's unique barcode for identification on microarrays, we have shown that this screening format is possible for enrichment screens (positive selection) (*24*) and lethality screens (negative selection) (*22,26*). Barcodes are essential for pool-based dropout screens, especially for, as example, those designed to identify cell lethal or drug sensitive shRNAs (*27*).

From our prostate cancer lethal screening efforts, an abundance of shRNAs targeting essential genes for cell survival and proliferation became reduced following cell passaging and thus "dropped-out" of the shRNA population. By comparing each shRNA's abundance in an initial cell population taken shortly after retroviral shRNA library infection to its abundance in samples taken after several cell population doublings, hundreds of lethal shRNAs were identified. Moreover, comparisons between the shRNA lethality profiles of our chosen prostate cancer cell lines (DU145, PC-3, LNCaP) with a more normal immortalized human prostate epithelial cell line (RWPE-1) resulted in the identification of numerous potential PCL genes.

We originally proposed to carry out genome-wide shRNA screens with two normal human prostate epithelial cells and four prostate adenocarcinoma cell lines that represent the diversity of prostate metastases which develop. We intended to engineer one of the normal prostate cell lines by immortalizing primary prostate epithelial cells (PrECs, orginally derived from the normal prostate of a 17-year old male (Clonetics)) with hTERT (*28*). Unfortunately, we attempted to create a stable hTERT-expressing immortalized PrEC cell line; however, the resultant clones were incapable of bypassing replicative senescence. For this reason, we focused our attention, at least temporarily, on the normal immortalized prostate epithelial cell line RWPE-1 for comparative analysis with the prostate cancer cell lines. RWPE-1 cells are a HPV-18 -immortalized prostate epithelial cell line (p53+, pRB+) molecularly engineered from the histologically normal prostate epithelia of a 54-year-old male (*29*).

From a list of prostate cancer cell lines commercially available, we selected DU145, PC-3, LNCaP, and MDA-PCa-2b. The DU-145 line is an androgen-independent cell line established from a prostatic metastasis of the brain (*34*) and is p53-, p16-, and pRb-mutated (*31*). The PC-3 adenocarcinoma cell line was obtained from a grade IV androgen-independent prostate cancer metastasis of the bone (*30*). It exhibits low 5α-reductase activity and is p53-, p16-, and pRb mutated (*31*). LNCaP-FGC cells were isolated from a prostatic metastasis of lymph nodes. They are androgen-dependent and wild-type for p53, p16, and pRb (*31*) and express mutated AR (*35*). The MDA-PCa-2b adenocarcinoma cell line was also derived from a bone metastasis of androgen-independent prostate cancer. These cells are p53+ and pRb+ (*32*) and have a mutated AR (*33*). RWPE-1 (cat# CRL11609), DU-145 (cat# HTB-81), PC-3 (cat# CRL-1435), LnCaP-FGC (cat#CRL-1740), and MDA-PCa-2b (cat # CRL-2422) cell lines were all purchased from ATCC. To date, screen data has been collected for all prostate cancer lines listed above except for MDA-PCa-2b cells.

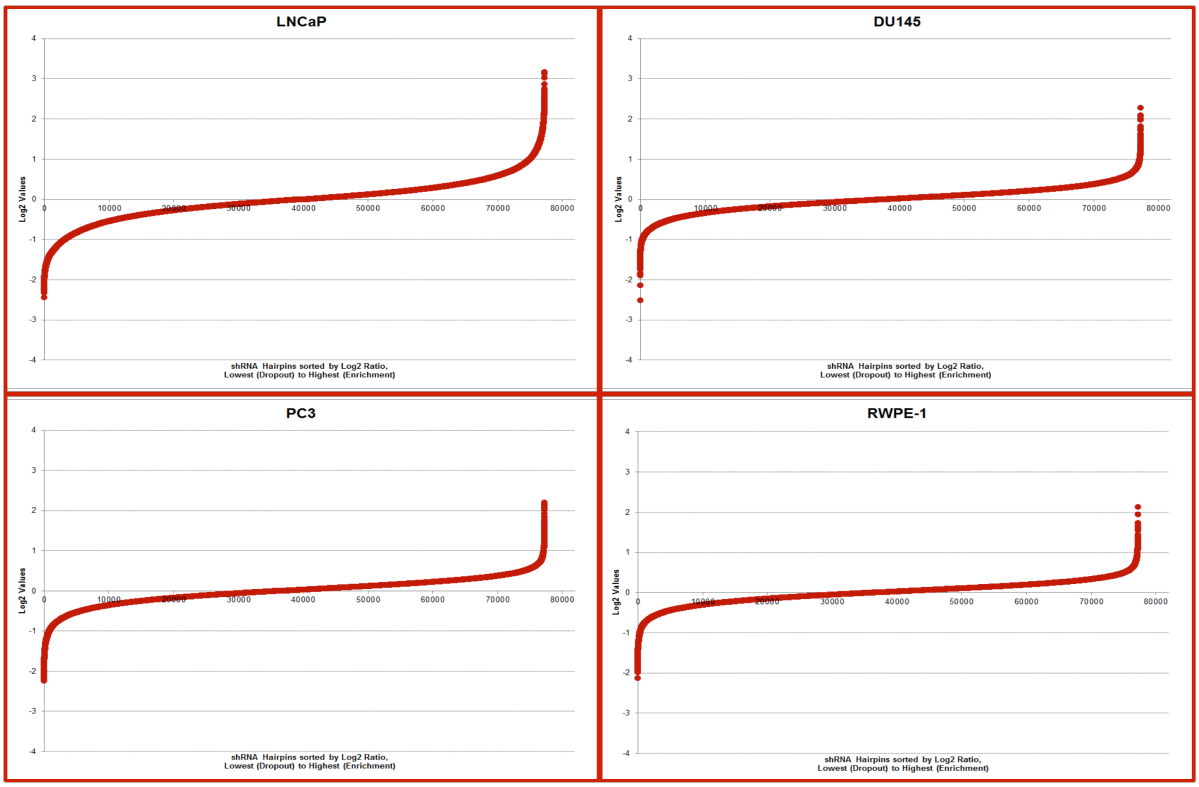
We conducted the prostate cancer lethal screening as previously described by this lab (22,26) in regards to other cancer lines, albeit with a few modifications. Briefly, cells were infected in triplicate with ~78,000 shRNAs targeting all coding sequences in the human genome at an average of 3 shRNAs per gene, and a representation of 1000 cells per shRNA at an MOI of 2. Initial reference samples were collected 72 hours post-infection, and the remaining cells were puromycin-selected and propagated with a representation of ≥1000 cells per shRNA maintained at each passage. Infected cells were

collected as the end samples after 8 population doublings (PDs). Cy3- and Cy5-labeled probes (from end or initial samples, respectively) were prepared and competitively hybridized to half-hairpin barcoded microarrays in order to measure the change in representation of each shRNA over time. Statistical analysis on similar lethality screens performed in our lab have determined that >90% of probes consistently yield signals >2-fold above the mean background of negative control probes across all triplicates, and the correlations among samples across triplicates and between the initial and end samples within each replica are high, signifying high reproducibility and maintenance of representation (*22*). These data were analyzed using a custom statistical package based on the LIMMA method (*36*) for the analysis of 2-color cDNA microarrays. We applied the method of significance analysis for microarrays (SAM) (*37*) with a false discovery rate (FDR) of 5% and a mean log2 ratio >1 (>2-fold depletion) to identify those shRNAs consistently depleted across the triplicates in each cell line. shRNAs with a difference in the mean log2 ratios >0.75 between the prostate cancer cell line and the normal line are considered as potential PCL genes.

Comprehensive genome-wide shRNA lethality screening and data analysis was thus completed for the normal prostate epithelial cell line RWPE-1 as well as for the prostate cancer cell lines DU-145, PC-3, and LNCaP. Log2 (Cy3/Cy5) ratios for each shRNA hairpin were highly reproducible across replicates. Similar to previous screens performed in breast cancer and colorectal cancer lines in this lab, a symmetrical distribution of "dropout" and "enrichment" hairpins was observed for all prostate cell lines screened (**Figure 2**). With the quality of the data within the prescribed limits, we compared the lethality profiles of each of the prostate cancer lines (PrCa) versus the normal prostate epithelial line RWPE-1.

Identifying PCL genes that are either common across all prostate cancer cell lines tested or unique to a subset of prostate cancer cell lines with similar pathologies may reflect common pathways required to support tumor growth and represent attractive drug targets. Thus, we performed a cluster analysis of all shRNA "dropouts" (log2 Cy3/Cy5 ratio < -1.0) identified in each of the prostate cell lines screened using the program Cluster 3.0 (© Stanford University (1998-1999)) (**Figure 3**). As expected, we observed marked differences between the PrCa cell lines and the normal prostate epithelial line RWPE-1. Many of these differences likely represent the sought-after PCL genes. Of the highest ranking PCL hairpins, based on absolute differences between PrCa and normal prostate epithelial cells (Δlog2 values = log2 ratio (PrCa) - log2 ratio (RWPE-1)), almost one-third are either putative nucleic acid binding proteins and/or transcription factors (**Figure 4**). This is consistent with the identification of numerous nuclear hormone receptor family members among our list of PCL candidate genes. Strikingly, seven (or 17.5%) out of the top forty PCL hairpins common to each of the three PrCa lines screened correspond to nuclear hormone receptors (**Figure 5**). Further, PCL hairpins corresponding to 22 out of a total 48 members of the nuclear hormone receptor family were identified for the DU145, PC-3, and LNCaP prostate cancer lines. This PCL candidate list includes the androgen receptor (AR) (*5*) and peroxisome proliferator-activated receptor-gamma (PPAR-gamma) (*52*), both of which have been previously implicated in prostate cancer disease progression.

A.



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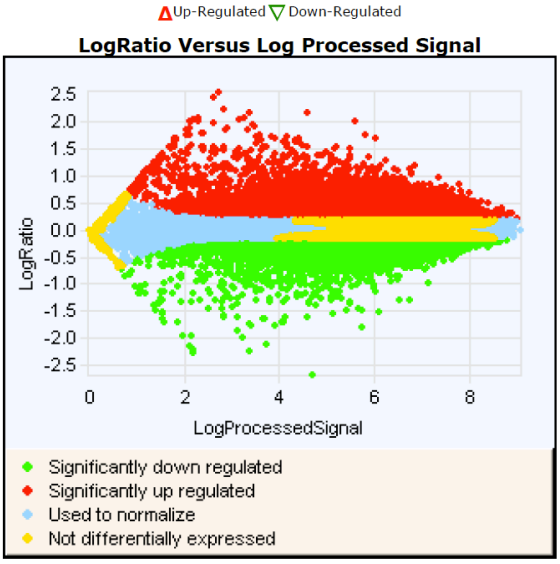

Figure 2. Ranked distribution (by log2 ratios) of shRNA hairpins in prostate cell lines.
A. Shown are the distributions of shRNA hairpins by relative representation (expressed as log2 [(PD=8)/(PD=0)]) in pre- vs. post-proliferative populations of prostate cancer (LNCaP, DU145, PC-3) and immortalized prostate epithelial (RWPE-1) cell lines, ranked lowest (dropouts) to highest (enrichments). Approximately symmetrical distributions were obtained for all four cell lines. (PD= population doublings) **B.** Representative distribution of shRNA hairpins by log2 ratios and hybridization signal strength for one of the replicates of the screen (example, DU145).

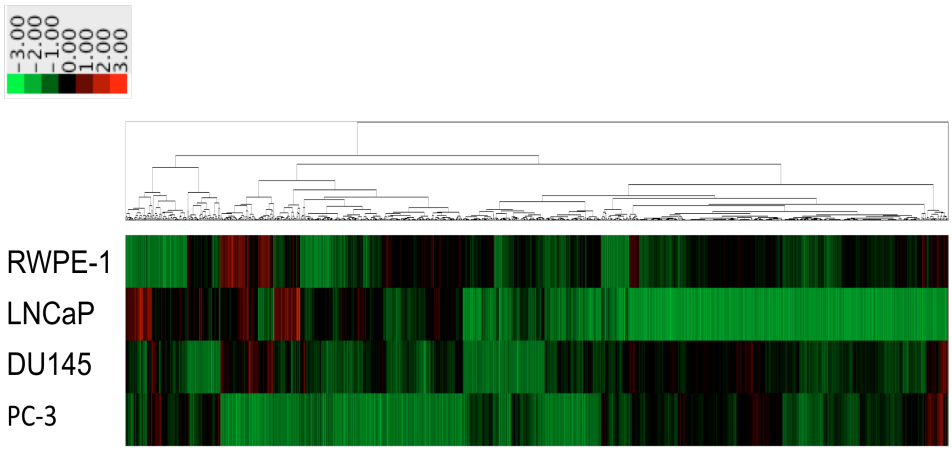

Figure 3. Cluster analysis of prostate cancer (PrCa) and immortalized prostate epithelial cell (PrEC) lines. Clustering of androgen-dependent (LNCaP) and –independent (DU145, PC-3) PrCa lines compared with "normal" immortalized (RWPE-1) nPrECs with respect to all antiproliferative shRNAs identified in the screen. Dropout shRNAs were arbitrarily set at a log2 Cy3/Cy5 ratio cutoff of < -1.0. The color scale represents mean normalized $\log_2$ Cy3/Cy5 ratios of the probes (dropouts in green; enrichment=red). Analysis performed with Cluster 3.0 (using C Cluster Library 1.50), © Stanford University (1998-1999).

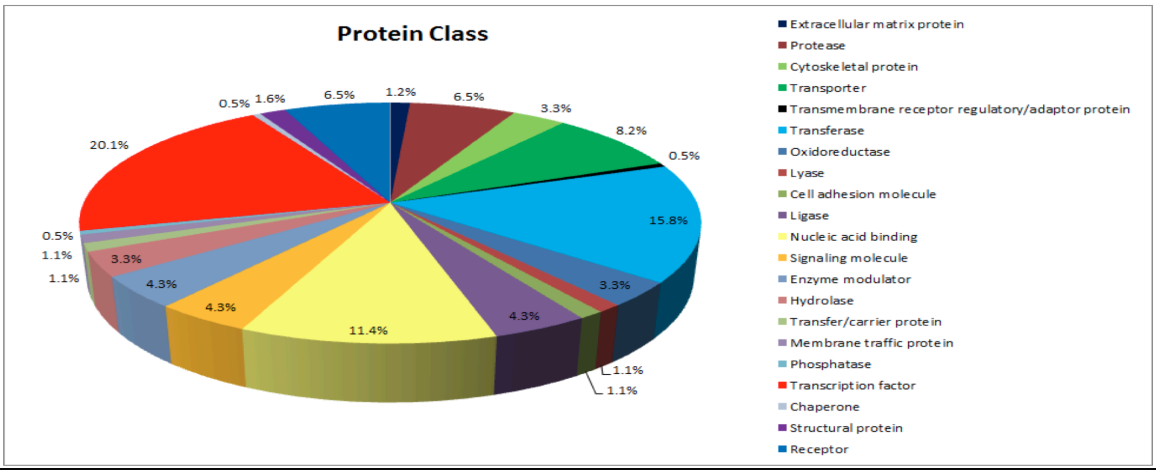

Figure 4. Analysis of hits from prostate cancer-specific lethal (PCL) screen.
The top 250 candidate PCL genes were parsed by gene ontology (GO) (A) Protein Class, (B) Molecular Function, and (C) Biological Process, using PANTHER (Protein ANalysis THrough EVolutionary Relationships) Database (www.pantherdb.org). Transcription factors (20.1%), transferases (15.8%), and nucleic acid binding proteins (11.4%) represent the top three categories of candidate PCLs.

Symbol	*PCL (DU145)*	*PCL (PC-3)*	*PCL (LNCaP)*	*Avg.*
ABAT	1.56	1.76	2.03	1.78
MAP3K9	1.20	1.60	1.24	1.35
POLR2E	1.36	1.37	1.16	1.30
ZNF143	0.91	1.62	1.30	1.28
NR4A2	1.00	1.25	1.56	1.27
GRIPAP1	1.18	1.29	1.29	1.25
CAMKK1	0.86	1.10	1.72	1.23
MPZ	1.40	0.83	1.42	1.22
MED26	1.46	0.80	1.39	1.22
GZMB	1.15	1.00	1.45	1.20
TM2D2	1.10	1.02	1.38	1.17
NR1H3	1.19	0.93	1.35	1.16
NR1I3	0.77	1.16	1.51	1.15
PODXL2	1.14	1.16	1.13	1.14
CTSB	1.08	1.34	0.91	1.11
BRS3	0.82	1.13	1.38	1.11
FBXO33	0.97	1.08	1.27	1.11
ANLN	1.06	0.89	1.34	1.10
UBR4	1.35	0.96	0.99	1.10
RARA	0.79	1.57	0.92	1.09
KCNK1	1.00	1.37	0.86	1.08
RXRA	0.92	1.30	1.00	1.08
NR2C2	0.89	1.35	0.98	1.07
PHF16	0.99	0.92	1.29	1.07
RPS7	0.84	0.97	1.29	1.03
PSMA4	1.07	0.83	1.19	1.03
HS3ST4	0.90	1.32	0.81	1.01
LOC340515	0.90	1.16	0.97	1.01
PKD2L1	0.82	0.83	1.37	1.00
RPS21	1.17	0.93	0.91	1.00
NTRK3	1.10	0.83	1.08	1.00
MFAP1	1.06	0.78	1.13	0.99
MPP1	0.93	1.09	0.91	0.98
CA9	0.90	0.97	1.03	0.97
LRRC32	0.75	0.76	1.30	0.94
TCEA1P	1.19	0.81	0.80	0.93
POLR2E	0.92	0.76	1.02	0.90
FBXL10	0.86	0.92	0.83	0.87
NR1I2	0.78	0.96	0.87	0.87
UBE1	0.77	0.86	0.77	0.80

Figure 5 Nuclear hormone receptor family members as potential PCL genes.
PCL hits common to DU145, PC-3, and LNCaP were ranked by their average Δlog2 values [log2 ratio (PrCa) - log2 ratio (RWPE-1)]. Seven of the top forty hairpins with the highest average Δlog2 values are shown to target distinct members of the nuclear hormone receptor gene family.
RARA = Retinoic acid receptor-alpha (NR1B1)
NR1I2 = Pregnane X receptor
NR1I3 = Constitutive androstane receptor
NR1H3 = Liver X receptor -alpha
RXRA = Retinoid X receptor-alpha (NR2B1)
NR2C2 = Testicular receptor 4 (TR4)
NR4A2 = Nuclear receptor related 1 (NURR1)

Validation of candidate PCL genes

Due to the shear number of potential PCL genes across the three prostate cancer cell lines screened (DU145, PC-3, and LNCaP), we have elected to perform a sublibrary screen of the top scoring hits. For each gene, twelve new shRNAs, each targeting unique sequences, have been designed and incorporated into a sublibrary pool for subsequent screening. In addition, a number of control hairpins have been added to this sublibrary to provide the baseline by which to distinguish true "dropouts" (PCL genes) from enrichments. For most genes in the original ~78K shRNA screen, only an average of 3-4 hairpins were represented per gene. Screening a sublibrary with twelve shRNA hairpins per potential PCL gene promises to be a more comprehensive and rigorous approach to validation than what we had originally proposed. At this time, the PCL candidate sublibrary of shRNA sequences has been synthesized and cloned into the original MSCV backbone upon which the original shRNA library was engineered. It is currently being spot-checked and will subsequently be packaged into retrovirus for subsequent screening.

After screening the PCL sublibrary and narrowing our candidate list, we will perform secondary validation assays using additional independent shRNAs against the target genes. To validate each gene, PrEC-hTERT E156T, RWPE-1, PC-3, DU-145, and LNCaP cells will be plated in 96-well plates and individually transduced with single shRNAs. Cell viability will be assessed several days later with the CellTiter-Glo Assay. We will also assess the effect of individual shRNAs on normal prostate and prostate cancer cells using the multi-color competition assay (MCA) (*38*). siRNAs will additionally be used for further validation. PCL candidates that also validate in the secondary assays will have shRNA and siRNA knockdown efficiency in the normal and cancer cell lines determined using RT-PCR and Western blotting. Rescue experiments with shRNA-resistant cDNAs must also be performed to ensure that cDNA expression of the target gene reverses the effect of the shRNA on cell viability. Only those genes corresponding to positive rescue experiments results will be considered further for mechanistic studies.

Bioinformatic analysis to identify candidates for future mechanistic studies.

One significant challenge associated with large scale screens is prioritization of candidate genes. We expect to identify a group of PCL genes, which are required for viability for some of the lines while others will be required for viability of all prostate cancer lines but not normal prostate epithelial cells tested. To prioritize candidate genes for further study, we will concentrate first on candidate genes that score with multiple shRNAs in the original screen. Moreover, available databases of genomic, transcriptional, functional, and protein-protein interaction information will be utilized to identify potential oncogenes by searching for genes amplified (*39,40*), mutant (*41,42*), or overexpressed in these cell lines and human breast cancers (*39,43*). Because we expect that some genes will fall into previously identified signaling pathways, we will also employ gene ontology (GO) categories (*44,45*), protein-protein interactions and Ingenuity analysis (*46*) to identify signaling networks that might connect genes that are not prioritized by the above criteria.

Future Proposed Work

Determination of the mechanisms of action of validated candidate genes whose loss of function selectively impair prostate cancer cell viability.

shRNAs that either reduce cell proliferation rate or increase the death rate should cause, theoretically, a decrease in cell number. We will use propidium iodide (PI) staining and FACS analysis to determine cell cycle profiles, investigate cell cycle arrest, and investigate whether an increase in the sub-G1 (apoptotic) population is observed upon shRNA expression. TUNEL staining and BrdU incorporation assays will be also performed to determine if candidate shRNAs reduce viability by increasing apoptosis or decreasing proliferation, respectively. Based on literature searches, protein domain information, and potential sites for protein modification, candidate genes will be further investigated to identify related signaling networks and the mechanism by which each candidate supports cancer cell proliferation and/or survival. Those that are required by a subset of prostate cancer cells, particularly the androgen independent cells, will be investigated for the potential perturbation of these oncogenic pathways.

Correlation of candidate gene expression, amplification or deletion with tumor grade and patient prognosis.

Accurately determining the *in vivo* prognostic significance of candidate genes is key to discovering potential drug targets, as well as in identifying possible biomarkers to predict disease progression. Tissue microarray slides (TriStar Technology Group, Rockville, MD) containing 2800 prostate cancer specimens with documented clinical histories will be separately immunostained with antibodies against individual candidate genes and counterstained with haematoxylin. Slides will be scanned and scored using the following scale: 0 – negative staining; 1 – weak staining, <50% of individual cell or <5% of all cells; 2 – moderate staining, >50% of individual cell or >5% of all cells (*47*). Tissue samples will be categorized based on histopathological factors, and the correlation significance will be determined using Pearson's chi-squared test. Kaplan-Meier plots will be constructed to establish an association between patient survival and candidate gene expression levels. The COX proportional hazards test for multivariate analysis will also be applied to exclude effects of other prognostic factors (tumor state, histological grade, etc.) on candidate gene expression. Candidates with a high correlation of gene expression levels and tumor grade and patient survival will represent strong candidates for further drug target and tumor diagnosis testing. Such analysis has already revealed strong prognostic significance of gene expression levels in prostate tumors (*47*) and numerous other cancers such as breast (*48*), lung (*49*) and melanoma (*50*).

Statement of Work
Year 1.

Task 1 (Months 1-3)
In the first 3 months, we will generate the PrEC + hTERT cell line using a blastocydin-selectable retroviral construct. We will characterize this cell line to ensure stable expression and activity of hTERT using the telomeric repeat amplification protocol (TRAP) assay. Telomere length of PrEC-hTERT cells compared to late passage PrEC cells will be determined using the terminal restriction fragment length (TRL) assay.

To date, we have attempted to engineer a stable hTERT-expressing normal PrEC cells; however, we have been unable to bypass replicative senescence in these cells to generate the required immortalized cell line for our studies. Since we could not engineer the required PrEC-hTERT cell lines, we acquired a putative hTERT-immortalized prostate epithelial cell line (E156T) (Kogan, et al. 2006) from Varda Rotter's laboratory at the Weizman Institute of Science in Israel. The extremely low population doubling rate in the absence of exogeneous androgen (testosterone) treatment makes screening this cell line infeasible. We are currently in the process of acquiring the proper DEA license for purchasing the quantities of testosterone required for the necessary culturing of these cells. As a result of these technical roadblocks, we have focused most of our attention on the HPV-18 –immortalized prostate epithelial cell line RWPE-1 for comparative analysis with the prostate cancer cell lines.

Task 2 (Months 3-15)
We will perform genome-wide shRNA lethality screens on PrEC-hTERT, RWPE-1, PC-3, DU-145, LNCaP-FGC, and MDA-PCa-2b cells to generate lethality profiles for each cell line.

Comprehensive genome-wide shRNA lethality screening and data analysis has been completed for the normal prostate epithelial cell line RWPE-1 and for the prostate cancer cell lines DU-145, PC-3, and LNCaP. Initially, owing to its unique media requirements, the RWPE-1 cell line could not be successfully screened until we modified our retroviral infection protocol to eliminate all traces of fetal bovine serum during the production of the shRNA library virus. We will take a similar approach for infecting the hTERT-immortalized E156T cell line once we obtain the proper DEA license for purchasing sufficient quantities of testosterone for cell culturing purposes.

While we have not yet screened all of the prostate cell lines proposed, the comparative analysis of a normal prostate epithelial cell line (RWPE-1) with three well-characterized prostate cancer lines (both androgen -dependent (LNCaP) and -independent (DU145,PC-3) lines) has yielded a wealth of information. Perhaps, most interestingly, in addition to the androgen receptor, a significantly large number of nuclear hormone receptor family members have been identified as potential PCL genes.

Year 2.

Task 3 (Months 13-24)
We will perform validation experiments with multiple individual shRNAs and siRNAs against candidate PCLs in CellTiter-Glo and MCA assays in order to confirm candidates that reduce cell growth and viability in prostate cancer cell lines, but do not affect normal prostate cell viability. We will also determine which candidate PCLs identified in the primary screen are false positives due to off-target effects.

Due to the shear number of potential PCL genes across the three prostate cancer cell lines screened (DU145, PC-3, and LNCaP), we have elected to perform a sublibrary screen of the top scoring hits. For each gene, twelve new shRNAs, each targeting unique sequences, have been designed and incorporated into a sublibrary pool for subsequent screening. In addition, a number of control hairpins have been added to this sublibrary to provide the baseline by which to distinguish true "drop-outs" (PCL genes) from enrichments (ie. tumor suppressors, etc). For most genes in the original ~78K shRNA screen, only an average of 3-4 hairpins were represented per gene. Screening a sublibrary with twelve shRNA hairpins per potential PCL gene promises to be a more comprehensive and rigorous approach to validation than what we had originally proposed. At this time, the PCL sublibrary of shRNA sequences has been synthesized and cloned into the original MSCV backbone upon which the original shRNA library was engineered. It is currently being spot-checked and will subsequently be packaged into retrovirus for subsequently screening applications.

Task 4 (Months 13-24)
For candidates that validate in the secondary assays, we will use RT-PCR and Western blotting to examine shRNA and siRNA knockdown efficiency. Only genes with a correlation between mRNA or protein knockdown and multiple shRNAs or siRNAs affecting cell viability in two or more prostate cancer cell types will be considered for further study.

This task awaits the completion of the PCL sublibrary screening that has been described in Task 3.

Task 5 (Months 18-24)
We will generate a list of validated PCL genes for further analysis and study from the data generated in tasks 3 and 4.

This task awaits the completion of the PCL sublibrary screening that has been described in Task 3.

Year 3. (Future Work)

Task 6 (Months 24-30)
We will perform bioinformatics analysis to identify potential oncogenes by searching for genes amplified, mutated, or overexpressed in these cell lines and human breast cancers. This will allow us to prioritize validated PCL candidates for further mechanistic studies. Gene ontology (GO) categories, protein-protein interactions and Ingenuity analysis will also be utilized to identify signaling networks that might connect genes within the list.

Task 7 (Months 24-30)
We will determine whether validated PCL genes negatively affect cell viability (increase apoptosis) or cell proliferation (decrease replication) by using propidium iodide staining, FACS analysis, TUNEL staining and BrdU incorporation assays.

Task 8 (Months 30-36)
We will determine expression levels of validated PCL genes in a spectrum of prostate cancer tissue samples using a tissue microarray. This will determine if there is a prognostic correlation between gene expression and tumor histopathology and patient survival, as well as help identify strong drug target candidates.

Key Research Accomplishments

1. Completion of a shRNA lethality profiles for the immortalized prostate epithelial cell line RWPE-1 and prostate cancer cell lines (LnCaP, DU145, and PC-3).
2. Comparative analysis of the lethality profiles for the prostate cancer cell lines against the "normal" RWPE-1 prostate epithelial cell line.
3. Identification of a subfamily of nuclear hormone receptors as potential prostate cancer-specific lethal (PCL) genes.
4. Synthesis of a comprehensive sublibrary of PCL hits for high throughput validation of PCL genes.

Reportable Outcomes

Not applicable at this time.

Conclusions

Comparative genome-wide shRNA screening identifies a large subfamily of nuclear hormone receptors as potential PCL genes

Comprehensive genome-wide shRNA lethality screening and data analysis has now been completed for the normal prostate epithelial cell line RWPE-1 as well as for the prostate cancer cell lines DU-145, PC-3, and LNCaP. Thus, we have been able to compare the lethality profiles of each of the prostate cancer (PrCa) lines against the normal prostate epithelial line RWPE-1. By performing cluster analysis of all shRNA "dropouts" (log2 Cy3/Cy5 ratio < -1.0) identified in each of the prostate cell lines screened, we were able to observe marked differences between the PrCa cell lines and the normal prostate epithelial line RWPE-1. Many of these differences likely represent the sought-after PCL genes. Of the highest ranking PCL hairpins, almost one-third are either putative nucleic acid binding proteins and/or transcription factors. Consistent with these results, seven (or 17.5%) out of the top forty candidate PCL hairpins common to each of the three PrCa lines screened correspond to nuclear hormone receptors. In an extension of these observations, PCL candidate hairpins representing 22 out of a total 48 members of the human nuclear hormone receptor family were identified from prostate cancer lines screened (DU145, PC-3, and LNCaP). This extensive list of PCL candidate includes the androgen receptor (AR) (*5*) and peroxisome proliferator-activated receptor-gamma (PPAR-gamma) (*52*) both of which have been previously implicated in prostate cancer disease progression.

hTert-immortalized prostate epithelial cell line requires modification of cell culture conditions for screening

We have attempted to engineer a stable hTERT-expressing normal PrEC cells; however, we have been unable to bypass replicative senescence in these cells to generate the required immortalized cell line for our studies. Since we could not engineer the required PrEC-hTERT cell lines, we acquired a putative hTERT-immortalized prostate epithelial cell line (E156T) (*51*) from Varda Rotter's laboratory at the Weizman Institute of Science in Israel. The extremely low population doubling rate in the absence of exogeneous androgen (testosterone) treatment makes screening this cell line infeasible. We are currently in the process of acquiring the necessary DEA license for purchasing the amounts of testosterone required for optimal proliferation of these cells in culture. As a result of these technical roadblocks, we have focused most of our attention on the HPV-18 –immortalized prostate epithelial cell line RWPE-1 for comparative analysis with the prostate cancer cell lines. Despite these issues, a large number of candidate PCL genes have already been identified by the genome-wide shRNA screening already performed.

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